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TECHNICAL MANUSCRIPT 69

ENZYMATIC FORMATION OF D-KYNURENINE

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ENZYMATIC FORMATION OF D-KYNURENINE

The work reported here was performed under Project 4B11-12-065, "Viral and Rickettsial Agent Research, "Task -02, "Viral and Rickettsial Laboratory Research." This material was originally submitted as manuscript 5104.

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Project 1C022301A067

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FOREWORD

This research was done by Dr. Higuchi while on a Secretary of the Army Fellowship in the laboratory of Professor Osamu Hayaishi of the Department of Medical Chemistry, Kyoto University, Kyoto, Japan.

ABSTRACT

The work of Kotake and Ito in 1937 indicated that D-tryptophan is converted to D-kynurenine in the rabbit. A study of the enzyme system responsible for this reaction showed that a dialyzed supernatant fraction (ca. 20,000g) prepared from homogenates of rabbit intestinal mucosa was capable of converting D-tryptophan in the presence of air, Mg^{++} (10^{-3} M), adenosine (5×10^{-4} M), and methylene blue (10^{-4} M) to stoichiometric amounts of D-kynurenine. The enzyme activity was evident only in parts of the small intestines corresponding to the terminal 30 per cent of the organ adjacent to the colon. The product was identified as kynurenine by paper chromatography, absorption spectrum, and quantitation of UV optical density measurements in agreement with aromatic amine assay by the Bratton-Marshall method. The D-configuration was assigned primarily on the basis of results of chromatography by the procedure of Price and Dodge (1956), which distinguishes the D- and L-isomers of kynurenine. Methylene blue appeared essential for the reaction; FAD, FMN, DPN, TPN, or ferricyanide could not replace the dye. Adenosine could be replaced by ATP, 5'AMP, and 3', 5'cycle AMP, but not by adenine. The presence of added catalase was stimulatory for kynurenine formation, whereas the presence of an H_2O_2 generating system (L-amino acid oxidase and L-leucine) did not substitute for the methylene blue. The presence of a DPNH-generating system markedly stimulated the reaction. The crude enzyme preparations which were employed yielded L-kynurenine from L-tryptophan but at a much lower rate than with D-isomer, L-tryptophan was an inhibitor of D-tryptophan oxidation.

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I. INTRODUCTION

The pathways of L-tryptophan metabolism in animal tissues have been studied extensively.[1,2] Among these, an important reaction is the formation of L-kynurenine from L-tryptophan through an oxygenase reaction mediated by the enzyme L-tryptophan pyrrolase. This enzyme has been demonstrated in mammalian livers as well as in bacteria. L-kynurenine is a key intermediate in tryptophan metabolism; for example, it is a precursor of the pyridine nucleotide coenzymes. The metabolic pathways involving L-kynurenine are indicated in Figure 1.

This report deals with studies on the enzymatic formation of D-kynurenine from D-tryptophan in mammalian tissues. Early nutritional work had shown that D-tryptophan is able to replace L-tryptophan in the animal diet. Undoubtedly, mechanisms exist in the animal for the racemization of D-tryptophan to the L-form. On the other hand, it has been known from the work reported by Kotake and Ito in 1937[3] that the rabbit, when fed D-tryptophan, excretes large amounts of D-kynurenine in the urine. This preservation of the D-configuration of the tryptophan molecule is quite interesting. Kotake and Ito showed further that rabbits injected, rather than fed, D-tryptophan yielded very little D-kynurenine. These results indicated that intestinal sites were involved, but a question of the possible role of intestinal microflora remained to be clarified.

Our studies have shown, however, that the enzymatic activity for the conversion of D-tryptophan to D-kynurenine resides in tissues in the region of the rabbit ileum and the reaction proceeds according to the over-all equation shown in B, Figure 1.

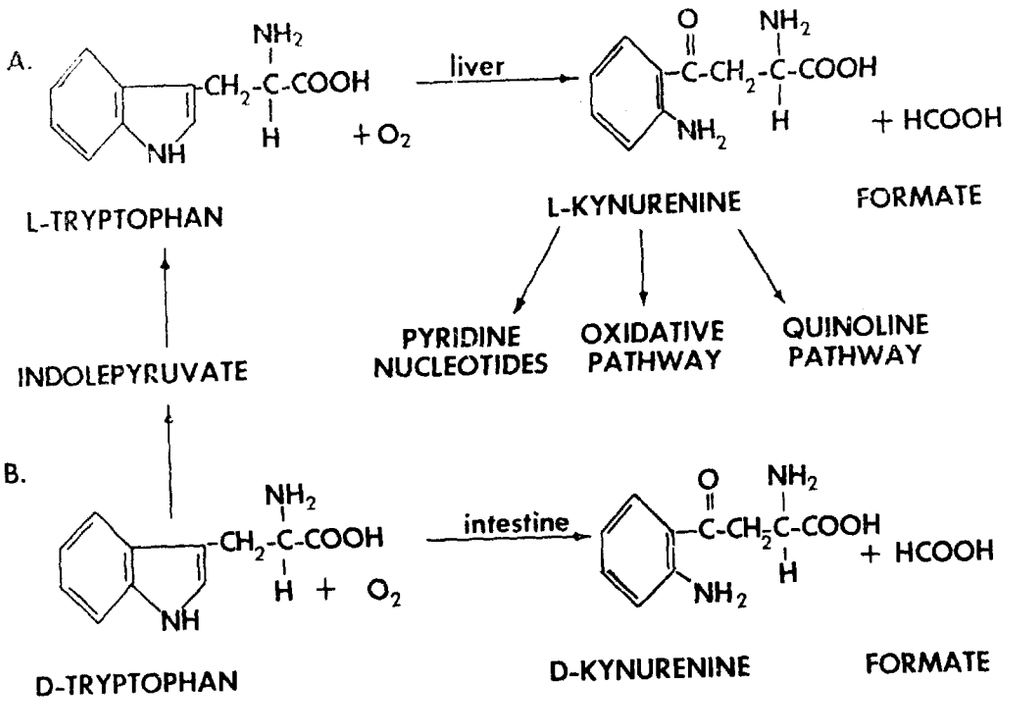

Figure 1. Metabolic Pathways of Optical Isomers of Tryptophan in Animals.

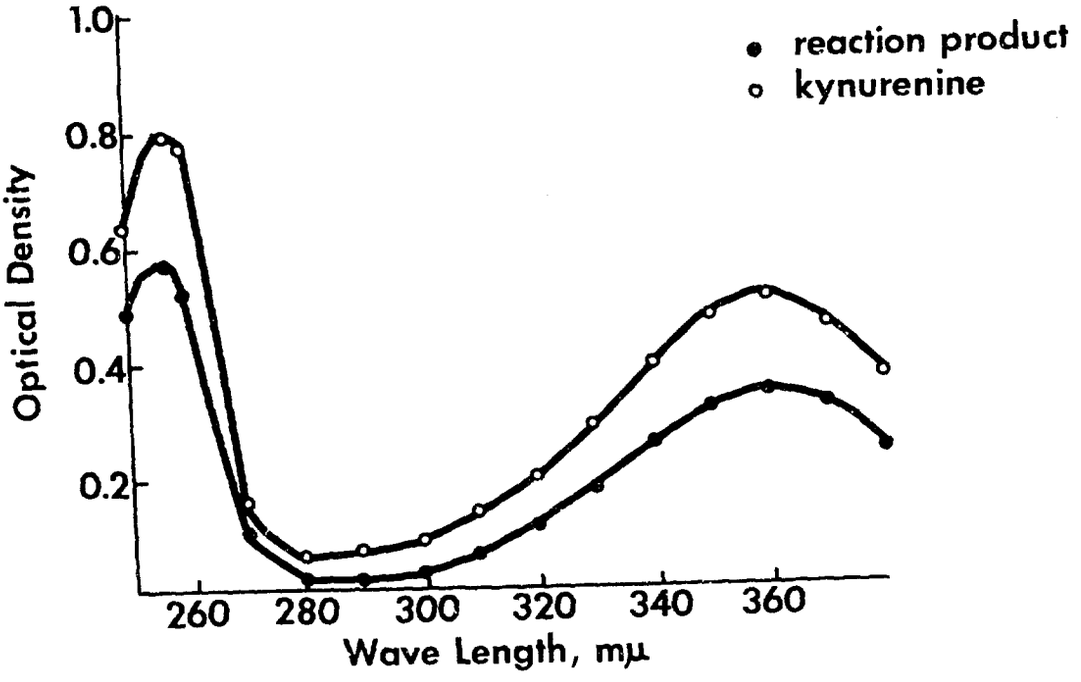

Figure 2. UV Absorption Spectra of Product of Reaction and Authentic Kynurenine.

II. MATERIALS AND METHODS

A. ENZYME PREPARATION

The experimental animal was the white domestic rabbit of approximately two kilograms body weight.* It was fed a slurry of two grams of D-tryptophan in water approximately five to six hours prior to sacrifice by exsanguination. The lower one-third of the small intestines was quickly removed, thoroughly washed in cold water, and a homogenate was prepared with two volumes of cold 0.1 M sodium phosphate, pH 7.0. The soluble fraction was obtained by centrifugation at 20,000g for 15 minutes. The supernatant was dialyzed in a cellophane bag for one and one-half hours against phosphate buffer (0.1 M), pH 7.0, containing 0.002 M EDTA and 0.001 M cysteine. This was followed by three buffer changes without the presence of EDTA. The cellophane bag contents were then recentrifuged at 20,000g and the supernatant was used in the enzyme studies. This material could be stored frozen without serious loss in activity for at least a week.

B. REACTION SYSTEM

The enzymatic reaction was usually conducted in 15 x 250 millimeter stoppered test tubes containing 0.4 milliliter of reaction mixture incubated aerobically in a constant-temperature bath at 36°C. The composition of the reaction mixture is presented in Table I.

TABLE I. EFFECTS OF OMISSION OF CONSTITUENTS OF ENZYME REACTION MIXTURE ON THE FORMATION OF D-KYNURENINE

REACTION SYSTEM	D-KYNURENINE YIELD, micromoles per milliliter per hour
Complete [a]	1.64
Complete minus methylene blue	0.00
Complete minus ATP	0.21
Complete minus Mg^{++}	1.11
Complete minus catalase	0.68

a. The complete system contained in a volume of 0.4 ml, in micromoles: methylene blue 0.04, ATP 0.40, Mg^{++} 0.40, Tris·HCl (pH 8.0) 40, D-tryptophan 2.0; catalase 60μg, and 0.1 ml of the enzyme preparation were also present.

* In conducting the research reported herein the investigators adhered to the "Principles of Laboratory Animal Care" as promulgated by National Society for Medical Research.

C. ASSAY

Kynurenine formation was measured spectrophotometrically in neutral phosphate buffer after deproteinization with perchloric acid. The Bratton-Marshall[4] chemical assay for the determination of aromatic amines was also employed for kynurenine determination.

III. RESULTS

As shown in Table I, the formation of D-kynurenine required methylene blue. Neither the pyridine nucleotide coenzymes nor the flavins (FAD and FMN) appeared able to replace the dye. The requirement for adenosine triphosphate ATP was satisfied by a variety of adenosyl compounds including adenosine, but not by adenine nor ribose. The presence of magnesium ions and catalase was also required for optimal reaction. Studies of these effects as well as of stimulation produced by the addition of a DPNH-generating system containing liver glucose-dehydrogenase will be presented in detail.

A. IDENTIFICATION OF D-KYNURENINE

The reaction product was identified as kynurenine by its UV absorption spectrum (Figure 2), its behavior on a Dowex-1-formate ion exchange column, and the Bratton-Marshall test for aromatic amines. The D-configuration was assigned on the basis of paper chromatography by the method of Price and Dodge, which distinguishes the D- and L-isomers (Figure 3).

B. STOICHIOMETRY

The stoichiometry of the reaction was obtained by determining the disappearance of D-tryptophan, the O_2 uptake, and the formation of D-kynurenine and formate. The time course of the reaction is plotted in Figure 4 and shows that the yield of D-kynurenine was essentially quantitative. The data from two such experiments, together with the amount of formate produced, are shown in Table II. Oxygen consumption in the reaction was measured in a separate experiment employing the Warburg manometer. Varying amounts of D-tryptophan were added to a series of six flasks. The oxygen consumption in these flasks, together with the yields of D-kynurenine, are presented in Table III. These data support the formulation of the reaction as the consumption of one mole of oxygen per mole of D-tryptophan to yield one mole each of D-kynurenine and formate.

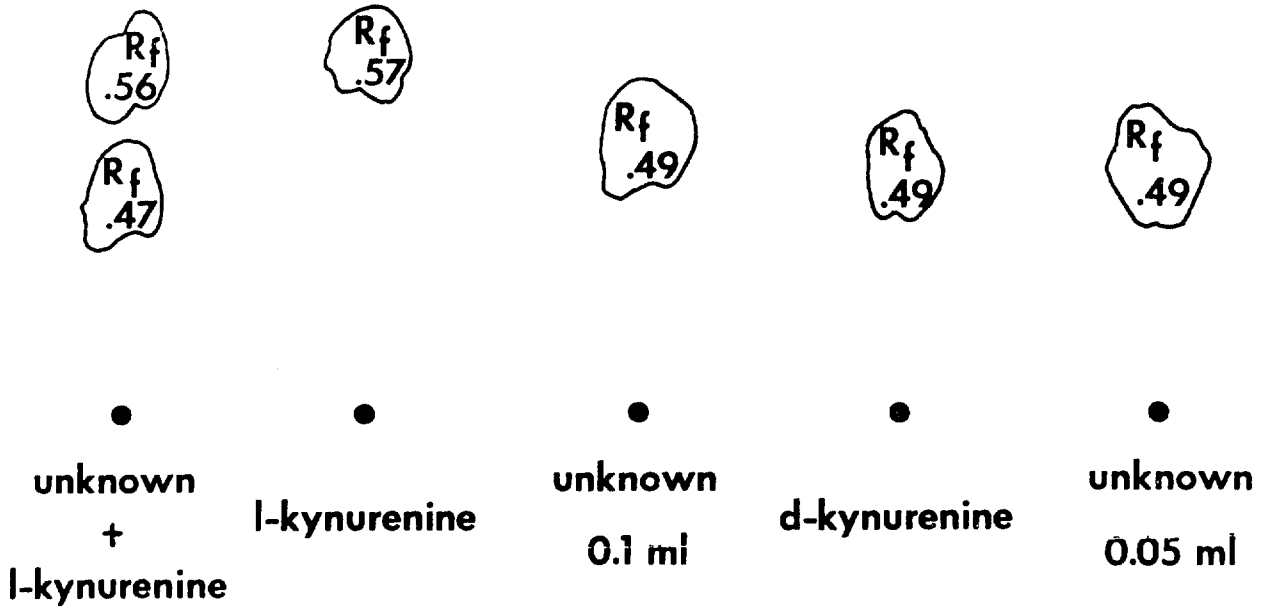

Figure 3. Identification of D-Kynurenine by Paper Chromatography.

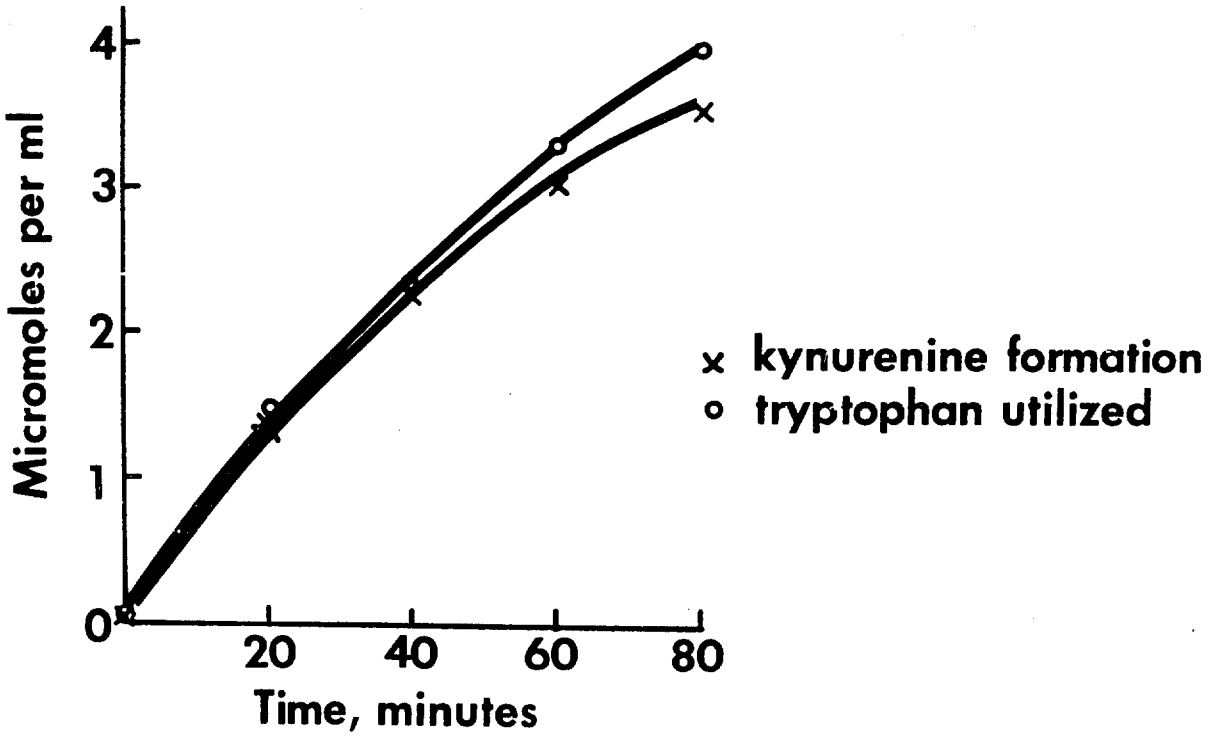

Figure 4. Time Course of D-Kynurenine Formation.

TABLE II. STOICHIOMETRY OF D-KYNURENINE FORMATION

SUBSTRATE AND PRODUCTS	μMOLES/ML [a]	
	A	B
D-tryptophan utilized	3.9	1.4
D-kynurenine produced	3.6	1.5
HCOOH formation	3.0	1.2

a. Reaction time 80 minutes. Tryptophan assayed by the method of Fishl,[5] and formate by the method of Grant.[6]

TABLE III. STOICHIOMETRY OF OXYGEN CONSUMPTION IN D-KYNURENINE FORMATION FROM D-TRYPTOPHAN

AMOUNT OF SUBSTRATE, μmoles/flask	D-KYNURENINE, μmoles/flask	O_2 UPTAKE [a] μmoles/flask
2	1.8	2.4
4	3.9	3.0
6	5.6	4.1
8	7.1	9.5
12	9.2	11.5

a. O_2 uptake values have been corrected for endogenous uptake of 4.3 μmoles.

C. pH OPTIMUM

The pH optimum for the reaction is approximately 8.0, as shown by the data plotted in Figure 5. Tris·HCl buffers at 0.1 M concentration were employed in the experiments.

D. METHYLENE BLUE CONCENTRATION CURVE

The effects of varying concentrations of methylene blue are shown in Figure 6. Approximately 2×10^{-5} M methylene blue is required for maximal D-kynurenine formation. Ferricyanide was unable to substitute for methylene blue. Other oxidation-reduction dyes were not tested.

E. DPNH GENERATING SYSTEM

D-kynurenine formation was markedly stimulated when a DPNH-generating system was added to the standard reaction mixture. A liver glucose dehydrogenase plus glucose and DPN constituted the generating system. The omission of any one of the three components of the generating system resulted in reduced kynurenine formation (Table IV).

F. ADENOSINE REQUIREMENT

The effects of varying concentrations of adenosine on the formation of kynurenine are shown in Figure 7. ATP and adenylic acid (5'AMP) also produced stimulation of the reaction equal to those obtained with adenosine at equivalent concentrations. Guanosine produced only partial stimulation even with increased concentrations. Adenine and the pyrimidine ribosides were totally ineffective. These studies were made in the standard system without the DPNH-generating system because the adenosine effect was absent in the presence of the pyridine nucleotides. The role of the adenosyl compounds in stimulating the reaction is at present obscure.

G. SUBSTRATE SPECIFICITY

The enzyme system employed in these studies was able also to attack L-tryptophan to a limited degree. L-kynurenine was produced at approximately one-fifth the rate for D-kynurenine formation. Purification of the enzyme is necessary before concluding whether two separate enzymes are involved. It was interesting to observe, however, that the presence of three micromoles per milliliter of the L-isomer appeared to completely inhibit the formation of D-kynurenine from D-tryptophan (Table V).

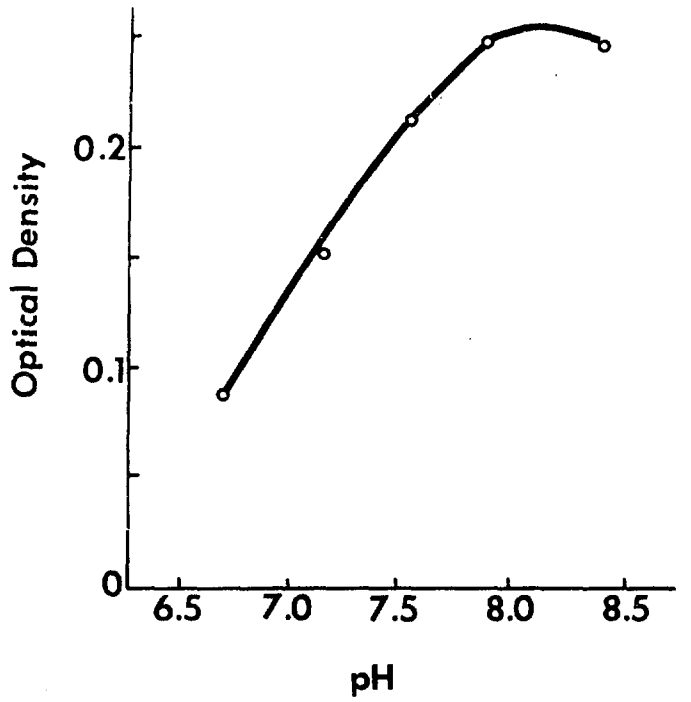

Figure 5. Effect of pH on Kynurenine Yield in 0.1 M Tris·HCl Buffer.

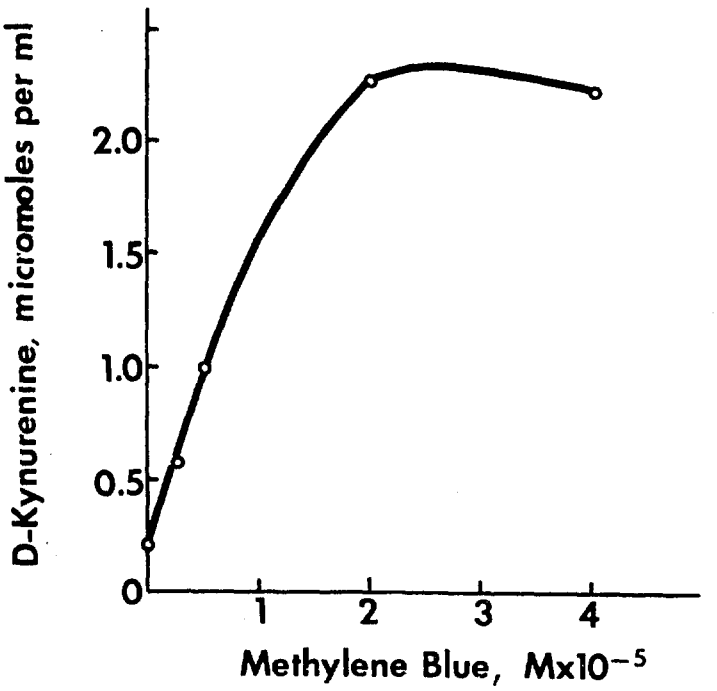

Figure 6. Effect of Methylene Blue on the Yield of D-Kynurenine.

TABLE IV. EFFECT OF A DPNH-GENERATING SYSTEM ON FORMATION OF D-KYNURENINE FROM D-TRYPTOPHAN

REACTION SYSTEM	D-KYNURENINE FORMATION, μmoles/ml/hr
Complete [a]	1.66
Complete minus substrate	0.00
Complete minus methylene blue	0.10
Complete minus enzyme	0.05
Complete minus glucose	0.60
Complete minus DPN	0.75
Complete minus glucose dehydrogenase	0.47

a. The complete system included the addition of a DPNH-generating system consisting of 60 micromoles of glucose, 0.2 micromoles of DPN, and 0.02 ml of a beef liver dehydrogenase preparation to the standard mixture.

TABLE V. INHIBITION OF D-KYNURENINE FORMATION BY L-TRYPTOPHAN

SUBSTRATE, μmoles/ml		KYNURENINE YIELD,
D-tryptophan	L-tryptophan	μmoles/ml
0	0	0.03
3	0	1.70
3	1	0.87
3	2	0.43
3	3	0.33
4.5	3	0.33
6	3	0.30
6	0	2.00
0	3	0.37

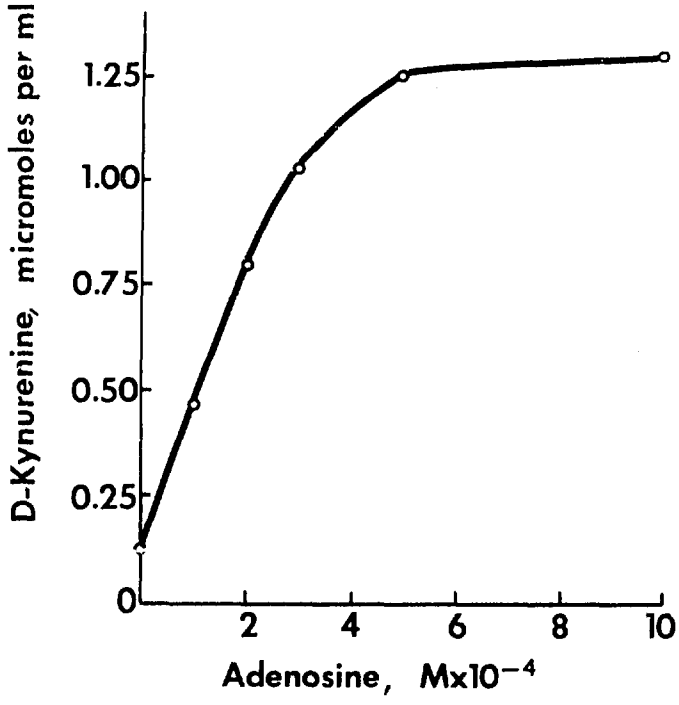

Figure 7. Effect of Adenosine on D-Kynurenine Formation.

IV. CONCLUSION

The data presented have shown that an enzyme system is present in the soluble fraction of homogenates of the rabbit ileum, which converts D-tryptophan to D-kynurenine. The stoichiometry showed the consumption of one mole of oxygen and the formation of one mole of formate per mole of D-kynurenine produced. The present system requires methylene blue, adenosine, Mg^{++}, catalase, and a DPNH-generating system for maximal kynurenine yields. An oxygenase reaction involving participation of reduced pyridine nucleotides is indicated. The enzyme (or enzymes) apparently differs from the L-tryptophan pyrrolase of mammalian liver in its mode of action. Its presence in the intestinal tissue presents an intriguing question as to its physiological role.

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